JOURNAL OF BACTERIOLOGY, June 1982, p. 1098–1102
0021-9193/82/061098-05$02.00/0

Vol. 150, No. 3

Cation-Dependent Binding of Substrate to the Folate Transport Protein of *Lactobacillus casei*

GARY B. HENDERSON* AND SUZANA POTUZNIK

Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037

Received 20 October 1981/Accepted 18 January 1982

Lactobacillus casei cells grown in the presence of limiting folate contained large amounts of a membrane-associated binding protein which mediates folate transport. Binding to this protein at 4°C was time and concentration dependent and at low levels (1 to 10 nM) of folate required 60 min to reach a steady state. The apparent dissociation constant (K_d) for folate was 1.2 nM at pH 7.5 in 100 mM K-phosphate buffer, and it varied by less than twofold when measured over a range of pH values (5.5 to 7.5) or in buffered salt solutions of differing ionic compositions. Conversely, removal of ions and their replacement with isotonic sucrose (pH 7.5) led to a 200-fold reduction in binding affinity for folate. Restoration of the high-affinity state of the binding protein could be achieved by the readdition of various cations to the sucrose medium. K_d measurements over a range of cation concentrations revealed that a half-maximal restoration of binding affinity was obtained with relatively low levels (10 to 50 μM) of divalent cations (e.g., Ca^{2+}, Mg^{2+}, and ethylenediammonium^{2+} ions). Monovalent cations (e.g., Na^+, K^+, and $Tris^+$) were also effective, but only at concentrations in the millimolar range. The K_d for folate reached a minimum of 0.6 nM at pH 7.5 in the presence of excess $CaCl_2$. In cells suspended in sucrose, the affinity of the binding protein for folate increased 20-fold by decreasing the pH from 7.5 to 4.5, indicating that protons can partially fulfill the cation requirement. These results suggest that the folate transport protein of *L. casei* may contain both a substrate- and cation-binding site and that folate binds with a high affinity only after the cation-binding site has been occupied. The presence of these binding sites would support the hypothesis that folate is transported across the cell membrane via a cation-folate symport mechanism.

Folate and structurally related compounds are transported into *Lactobacillus casei* via a single, high-affinity system which is regulated by the level of folate in the growth medium (3, 5, 10, 12). The process is energy dependent and can be classified as active since free folate can accumulate within the cells to levels that exceed the extracellular concentration by several thousandfold (7). The energy source for transport has been suggested from inhibitor studies to be an energy-rich phosphate compound, rather than a proton gradient or membrane potential generated by an energy-transducing ATPase (7). The transport system comprises at least two cellular components, a specific folate-binding protein (5, 6, 9) and a second factor that is also required for the transport of thiamine and biotin (8). The binding protein has been solubilized from the membrane and purified to homogeneity (6, 9). Distinguishing features of the binding protein are that it is present in relatively large amounts and can be easily quantitated in both intact cells and membrane preparations (5, 9). In the present study, cells depleted of energy reserves to prevent substrate transport (7) were employed to examine the interaction of folate with the binding protein. It was observed that the ability of the protein to bind folate is highly dependent upon the presence of cations in the external medium. The nature of this cation requirement suggests that folate uptake may proceed in *L. casei* via a cation-substrate cotransport mechanism.

MATERIALS AND METHODS

Radiolabeled folate. [3′,5′,9(*n*)-^{3}H]folate (500 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, Ill.) and purified by thin-layer chromatography on cellulose sheets (Eastman 6064), using 0.1 M K-phosphate (pH 7.0) as the solvent.

Growth of cells. *L. casei* subsp. *rhamnosis* (ATCC 7469) cells were grown in the presence of limiting amounts (2 nM) of folate for 16 h at 30°C as described previously (7).

Binding determinations. Folate-binding activity was measured in freshly grown cells that had been washed with 100 volumes of 100 mM K-phosphate (pH 7.5) and incubated for 60 min at 23°C to deplete energy

reserves (7). Assay mixtures consisted of [^{3}H]folate (250,000 dpm/nmol), the desired additions, and energy-depleted cells (7×10^8) in a final volume of 14.0 ml. After incubation at 4°C for 60 min (unless otherwise stated), the cells were collected by centrifugation at 32,000 × *g* (5 min, 4°C), suspended in 0.25 ml of water, and transferred to scintillation vials with the aid of two 4-ml samples of Cytoscint (Westchem Products, San Diego, Calif.). Controls were prepared identically, except that excess (100 μM) unlabeled folate was added before the [^{3}H]folate. After correction for the control (which was usually less than 10% of sample values), results were expressed in nanomoles of folate bound per 10^{10} cells. The dry weight of the latter was approximately 2.2 mg. Intracellular concentrations of folate were calculated from a cell volume of 2.4×10^{-12} ml (7).

RESULTS

Kinetics of folate binding. The uptake of folate by *L. casei* cells occurs in two distinct phases, a rapid binding of folate to the transport protein, followed by a much slower, glucose-dependent internalization of the vitamin (7). In the present study, the initial binding step was examined in the absence of substrate transport by performing the measurements at low temperature (5, 9) and by employing cells depleted of energy reserves (7). The rate of folate binding to energy-depleted cells (at 4°C) is shown in Fig. 1. At 25 nM folate, maximum binding was achieved within 10 min, whereas the time interval required to reach a steady state increased to 60 min when the folate concentration was reduced to 2 nM.

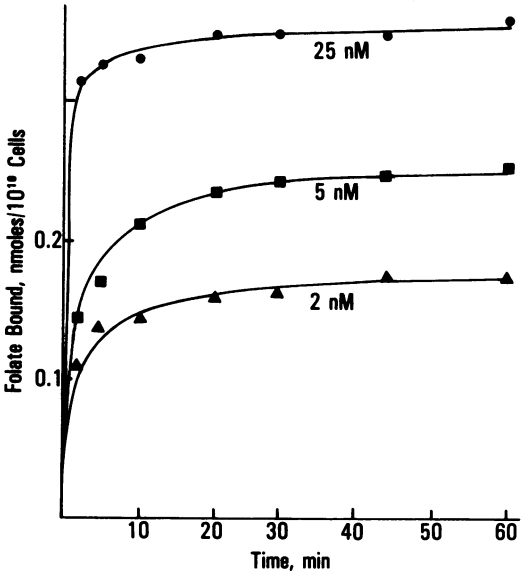

FIG. 1. Concentration dependence for the rate of folate binding at 4°C in energy-depleted cells. Bound substrate was determined (see the text) as a function of time at the indicated initial concentrations of folate. Suspending medium, 0.1 M K-phosphate, pH 7.5.

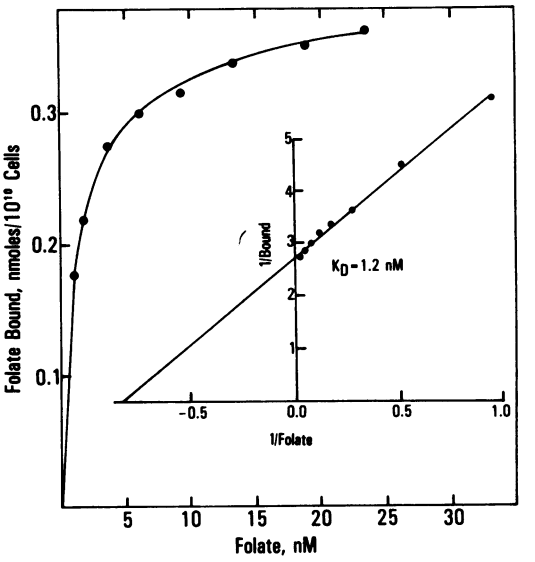

FIG. 2. Folate bound as a function of substrate concentration. Folate-binding activity was determined as described in the text and plotted as a function of the concentration of free (unbound) folate. Inset, double-reciprocal plot of bound versus free folate. Incubation conditions, 60 min at 4°C; suspending medium, 0.1 M K-phosphate, pH 7.5.

When folate-binding activity at the steady state (60 min, 4°C) was measured over a range of folate concentrations, the results shown in Fig. 2 were obtained. The ability to bind folate was a saturable process, and from a double-reciprocal plot of the data (inset, Fig. 2), the dissociation constant (K_d) for folate was determined to be 1.2 nM. Maximum binding activity was 0.37 nmol/10^{10} cells.

Effect of media composition on the K_d for folate. The above binding determinations were performed with cells suspended in 100 mM K-phosphate buffer, pH 7.5. When these same measurements were carried out with media of differing ionic compositions (pH 7.5), the K_d for folate was found to remain essentially unchanged (Table 1). Conversely, the binding protein of cells suspended in isotonic sucrose exhibited a substantial reduction (ca. 200-fold) in affinity for folate, although total binding activity was not affected by the transfer of cells to the sucrose medium (data not shown). When 100 mM KCl was added to cells that had been incubated for 60 min in sucrose (pH 7.5), the binding protein returned to its high-affinity state.

Effect of pH on the K_d for folate. K_d values measured in various buffered saline solutions as a function of pH are shown in Fig. 3. A minimum K_d for folate 1.2 nM was observed at pH 6.5, whereas progressively higher values were obtained as the pH was reduced to 4.5 or increased to 8.5. A substantially different pH profile was

TABLE 1. Effect of medium composition on the affinity of the binding protein for folate[a]

Medium composition (pH 7.5)	Concn (mM)	K_d (nM)
K-Phosphate	100	1.2
KCl	100	1.6
NaCl	100	1.9
Tris-chloride	100	2.5
Sucrose	250	300
Sucrose plus KCl	250	1.0
	100	

[a] Cells were suspended in the indicated solutions (pH 7.5) and incubated for 60 min at 4°C in the presence of variable concentrations of folate. The K_d for folate was then calculated from a double-reciprocal plot of bound versus free folate.

observed in cells suspended in 250 mM sucrose (Fig. 3). In the latter case, the K_d for folate exhibited a minimum at the lowest pH tested (4.5) and increased at higher pH values.

Cation dependence for folate binding. The data in Table 1 show that the binding protein of cells suspended in sucrose, which exhibits a low affinity for folate, can be restored to a high-affinity state by the addition of 100 mM KCl. To assess the concentration dependence of this change in affinity, K_d values for folate were measured in the presence of various amounts of added KCl. It was observed (Fig. 4) that millimolar concentrations of KCl were required to substantially increase the affinity of the protein for folate. NaCl was comparable to KCl in this respect, whereas $CaCl_2$ and $MgCl_2$ were effective at much lower concentrations. When excess (4 mM) $CaCl_2$ or $MgCl_2$ was present in the incubation medium, the K_d for folate decreased to 0.6 nM (from 1.2 nM in K-phosphate buffer).

The differential effects observed with the chloride salts of K^+, Na^+, Ca^{2+}, and Mg^{2+} showed that cations were the important species in promoting the binding of folate and that divalent cations were much more effective than monovalent cations. The relative effectiveness of these ions was quantitated by comparing cation levels required to lower log K_d to one-half the sum of log K_d (maximum) and log K_d (minimum). The latter concentration was calculated (at pH 7.5) to be 13.5 nM from the binding affinity for folate in cells suspended in sucrose alone (K_d = 300 nM) and in sucrose plus 4 mM $CaCl_2$ (K_d = 0.6 nM). The data in Fig. 4 show that Ca^{2+} and Mg^{2+} produced this half-maximal increase in affinity at concentrations of 14 and 50 μM, respectively, whereas the level of K^+ required to exhibit the same half-maximal effect was 10 mM.

From K_d measurements as a function of cation concentration (Fig. 4), various other organic and

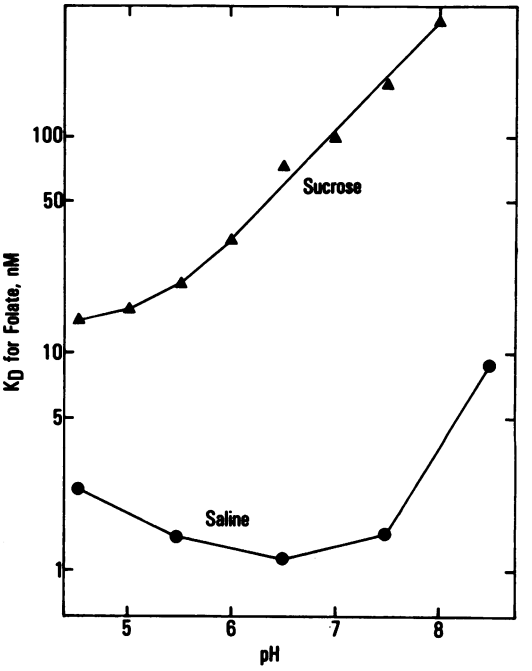

FIG. 3. pH dependence of the dissociation constant (K_d) for folate. K_d values were determined (see inset, Fig. 2) in either 250 mM sucrose adjusted to the indicated pH with HCl or KOH or in 100 mM KCl containing the following buffer solutions: 20 mM K-acetate (pH 4.5 and 5.5); 20 mM K-phosphate (pH 6.5 and 7.5); and 20 mM K-bicarbonate (pH 8.5).

inorganic monovalent and divalent cations were evaluated for the ability to enhance the affinity of the binding protein for folate. It was observed (Table 2) that divalent cations of diverse structure were each effective in the 10 to 50 μM

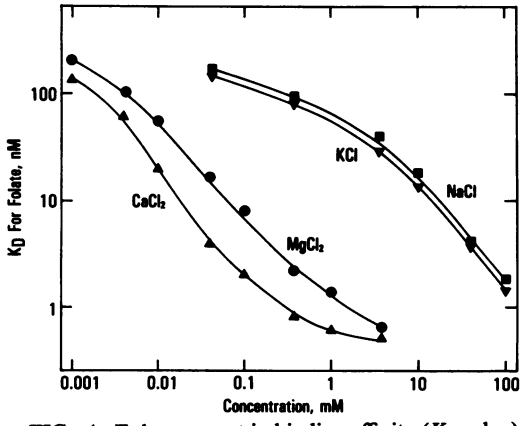

FIG. 4. Enhancement in binding affinity (K_d value) for folate as a function of cation concentration in cells suspended in isotonic sucrose. K_d values were determined as described in the legend to Fig. 2 in cells suspended in 250 mM sucrose (pH 7.5) containing the indicated additions.

TABLE 2. Relative effectiveness of cations in restoring the high-affinity state of the binding protein[a]

Cation	Concn (μM) for half-maximal binding affinity for folate
K^+	10,000
Na^+	13,000
NH_4^+	25,000
Ethylammonium$^+$	14,000
Tris$^+$	25,000
Triphenylmethylphosphonium$^+$	7,000
Mg^{2+}	50
Ca^{2+}	14
Zn^{2+}	32
Mn^{2+}	34
Ethylenediammonium^{2+}	29

[a] The cation concentration required to give a half-maximal affinity (K_d) for folate was determined from a plot (Fig. 4) of K_d versus the concentration of the chloride salt of the indicated cation. Half-maximal binding affinity is defined as the K_d for folate at which its log is equal to one-half the sum of log K_d (maximum) and log K_d (minimum). Under the conditions employed, the K_d for folate exhibited a maximum of 300 nM in sucrose alone and a minimum of 0.6 nM in sucrose plus 4 mM $CaCl_2$ and thus was half maximal when it reached a value of 13.5 nM. Suspending medium, isotonic sucrose, pH 7.5.

range, whereas monovalent anions invariably showed a half-maximal effect at levels that exceeded 5 mM.

The cation dependence of the K_d for folate was also assessed at pH 5.5. Employing Mg^{2+} and K^+ as representative cations, a half-maximal increase in binding affinity was achieved at concentations of $MgCl_2$ and KCl (42 μM and 10 mM, respectively) which were essentially the same as those obtained at pH 7.5 (Table 2). In this case, K_d values for folate ranged from 30 nM in sucrose alone to 0.5 nM in sucrose plus 4 mM $MgCl_2$, and a half-maximal affinity was represented by a K_d value of 4.0 nM.

DISCUSSION

L. casei cells contain relatively large amounts of a folate-binding protein that is a functional component of the folate transport system (5, 6, 9). This protein is an unusually hydrophobic, integral membrane component (5, 6, 9), and thus is probably also the carrier of folate into the cell. The high affinity of this protein for folate (K_d = 1 nM) allows the cells to bind the vitamin when it is present in trace amounts in the environment, although this efficient binding step is partially offset by the exceedingly slow rate of folate transport (0.8 molecules per min per binding site at 30°C). The folate transport system of *L. casei* thus conforms to an emerging pattern that transport proteins with a high affinity for a given substrate mediate substrate transport at a relatively slow rate (14). This property may be related to the difficulty of unloading substrates from high-affinity carrier proteins when their binding sites are exposed to the inner membrane surface (13).

The folate transport protein of *L. casei* exhibits a relatively usual property; it can be quantitated in intact cells without the complication of substrate transport into the cell interior. The ability to separate binding from transport has been possible because of a combination of various properties of this system. Of primary importance is the fact that the binding protein is present in large amounts per cell (5, 6, 9) and exhibits a very high affinity for folate (Fig. 2 and Table 1). This enables binding measurements to be performed under conditions in which the specific association of substrate with the cells is usually 10-fold greater than the nonspecific component. Moreover, with this high level of binding activity, the potential contribution by an energy-independent equilibration of folate across the membrane would be negligible (less than 1% relative to total uptake) at the extracellular folate concentrations (1 to 50 nM) employed in these binding measurements. The second major factor has been the ability to block the energy-dependent transport of folate by lowering the temperature to 4°C (5) and by using cells depleted of energy reserves (7). This lack of folate transport is indicated in the present study (Fig. 1) by the absence of a time-dependent uptake component subsequent to the initial binding step. A close correlation has also been established between the folate-binding activity in intact cells and the amount of Triton-extractable binding protein (5).

Measurements in the present study have demonstrated that the folate transport protein exhibits a high and relatively constant affinity for folate when the cells are suspended in buffers of various pH values (Fig. 3) and ionic compositions (Table 1). Conversely, removal of ions and their subsequent replacement with isotonic sucrose leads to a substantial reduction (200-fold at pH 7.5) in substrate-binding affinity (Table 1 and Fig. 3). The protein is not irreversibly denatured under these conditions since the high-affinity state can be restored by the readdition of various ions to the medium. A comparison of the chloride salts of various cations showed that the restoration process is specific for cations and that divalent cations such as Ca^{2+}, Mg^{2+}, and the ethylenediammonium^{2+} ion are effective at 100-fold-lower concentrations than K^+, Na^+, or other monovalent cations (Table 2 and Fig. 4). A variety of divalent cations were shown to be similar in their ability to promote folate binding, suggesting that the activation process is depen-

dent primarily upon the charge and not the size of cations. It is concluded from these results that cations may have a direct role in the binding and thus also the transport of folate into *L. casei*.

A possible role for cations in folate transport could be to maintain electroneutrality during the uptake process. Folate compounds and other divalent anions must enter bacterial cells against a substantial membrane potential (interior negative). The latter exceeds −150 mV in both *L. casei* (7) and other bacterial cells (1, 2, 11) and thus would represent a force which could potentially prevent folate compounds from crossing the cell membrane. One method for neutralizing the effect of the membrane potential would be to mediate an obligatory exchange of intracellular folate compounds for intracellular anions of equal charge, a process that has been suggested to occur in L1210 mouse cells (4). Electroneutrality could also be maintained by the simultaneous uptake of folate along with either a divalent cation or two monovalent cations. In molecular terms, this could be achieved if the carrier protein had functional binding sites for both folate and cations. If the latter occurs in *L. casei*, then the role of cations in folate uptake would presumably be restricted to balancing charge movement across the membrane and would not extend to the energy-coupling step since this transport system does not appear to be driven by an ion gradient (7). Protons can promote folate binding at low pH (Fig. 3), although the binding preference for other cations would suggest that proton-folate cotransport does not occur to a significant extent even at pH 4.5.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants CA32261 and CA6522 from the National Cancer Institute, by grant CH-31 from the American Cancer Society, and by grant PCM 77-23414 from the National Science Foundation.

We are indebted to F. M. Huennekens and K. S. Vitols for assistance in the preparation of the manuscript.

LITERATURE CITED

1. **Friedberg, I., and H. R. Kaback.** 1980. Electrochemical proton gradient in *Micrococcus lysodeikticus* cells and membrane vesicles. J. Bacteriol. **142**:651–658.
2. **Harold, F. M., and D. Papineau.** 1972. Cation transport and electrogenesis by *Streptococcus faecalis*. I. The membrane potential. J. Membr. Biol. **8**:27–44.
3. **Henderson, G. B., and F. M. Huennekens.** 1974. Transport of folate compounds into *Lactobacillus casei*. Arch. Biochem. Biophys. **164**:722–728.
4. **Henderson, G. B., and E. M. Zevely.** 1981. Anion exchange mechanism for transport of methotrexate in L1210 cells. Biochem. Biophys. Res. Commun. **99**:163–169.
5. **Henderson, G. B., E. M. Zevely, and F. M. Huennekens.** 1976. Folate transport in *Lactobacillus casei*: solubilization and general properties of the binding protein. Biochem. Biophys. Res. Commun. **68**:712–717.
6. **Henderson, G. B., E. M. Zevely, and F. M. Huennekens.** 1977. Purification and properties of a membrane-associate, folate-binding protein from *Lactobacillus casei*. J. Biol. Chem. **252**:3760–3765.
7. **Henderson, G. B., E. M. Zevely, and F. M. Huennekens.** 1979. Coupling of energy to folate transport in *Lactobacillus casei*. J. Bacteriol. **139**:552–559.
8. **Henderson, G. B., E. M. Zevely, and F. M. Huennekens.** 1979. Mechanism of folate transport in *Lactobacillus casei*: evidence for a component shared with the thiamine and biotin transport systems. J. Bacteriol. **137**:1308–1314.
9. **Henderson, G. B., E. M. Zevely, R. J. Kadner, and F. M. Huennekens.** 1977. The folate and thiamine transport proteins of *Lactobacillus casei*. J. Supramol. Struct. **6**:239–247.
10. **Huennekens, F. M., K. S. Vitols, and G. B. Henderson.** 1978. Transport of folate compounds in bacterial and mammalian cells. Adv. Enzymol. **47**:313–346.
11. **Padan, E., D. Zilberstein, and H. Rottenberg.** 1976. The proton electrochemical gradient in *Escherichia coli* cells. Can. J. Biochem. **63**:533–541.
12. **Shane, B., and E. L. R. Stokstad.** 1975. Transport and metabolism of folates by bacteria. J. Biol. Chem. **150**:2243–2253.
13. **West, I. C.** 1980. Energy coupling in secondary active transport. Biochim. Biophys. Acta **604**:91–126.
14. **Wilson, D. B.** 1978. Cellular transport mechanisms. Annu. Rev. Biochem. **47**:933–965.